

METHODS AND TRANSGENIC MOUSE MODEL FOR IDENTIFYING
AND MODULATING FACTORS INVOLVED IN THE PRODUCTION OF
REACTIVE OXYGEN INTERMEDIATES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Patent
Application Serial No. 60/395,498 filed July 12, 2002.

FIELD OF THE INVENTION

The present invention relates generally to the field of cellular
proliferation and more specifically to a non-human animal model for
overexpression of the *gp91*/phox homologue family of nicotinamide adenine
dinucleotide phosphate-reduced form (NADPH) oxidase (NOX) and dual
oxidase (Duox) enzymes and generation of reactive oxygen intermediates.

BACKGROUND OF THE INVENTION

Reactive oxygen intermediates (ROI), which include the
superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), result from the
stepwise, monovalent reduction of oxygen: O_2^- results from the addition of a
single electron to O_2 , and H_2O_2 results from the addition of a single electron to
 O_2^- . ROI are cytotoxic and mutagenic, with high levels of ROI causing
damage to biomolecules such as DNA, proteins, and biomembranes.
However, recent data indicate that lower levels of ROI may function in signal
transduction as intracellular mediators of cell growth, cell proliferation,
angiogenesis, apoptosis, and senescence.

Several biological systems generate ROI. For example, within
the phagocytic-based immune defense against invading microbes, cells such
as neutrophils produce large quantities of ROI via the nicotinamide adenine
dinucleotide phosphate-reduced form (NADPH) oxidase (also known as the

respiratory burst oxidase). The catalytic subunit of this enzyme, gp91*phox*, oxidizes NADPH and reduces oxygen to form O₂⁻.

In many non-phagocytic cell types including cells in the colon, lung, brain and kidney, the gp91*phox* homologue family of NADPH oxidase (NOX) and dual oxidase (Duox) enzymes is responsible for producing low levels of ROI. At present, six human homologues of gp91*phox* have been identified, with additional homologs present in rat, mouse, *Caenorhabditis elegans*, and *Drosophila*.

Although the functions of NOX and Duox-derived ROI are unclear, several studies suggest that the non-phagocytic generation of ROI modulates cellular proliferation and activation of growth-related signaling pathways. For example, both fibroblasts and endothelial cells produce increased levels of superoxide in response to cytokines such as interleukin-1 and tumor necrosis factor (TNF). In rat vascular smooth muscle cells, exposure to platelet-derived growth factor (PDGF) increases the release of H₂O₂ while concomitantly increasing cell proliferation (Meier *et al.*, Biochem. J. 263:539-45 (1989); Matsubara *et al.*, J. Immun., 137:3295-98 (1986)). Additionally, data show that low levels of ROI elicit downstream effects on the redox-sensitive transcription factor nuclear factor kappa-B (NFκ-B) and activator protein-1 (AP-1) (Schreck *et al.*, EMBO J., 10:2247-58 (1991); Schmidt *et al.*, Chemistry & Biology, 2:13-22(1995)).

Non-phagocytic ROI appear to have a direct role in regulating cell division, and may function as mitogenic signals in pathologic conditions related to cell growth, such as cancer and cardiovascular disease. For example, cytokine-mediated endothelial production of O₂⁻ may play a role in angiogenesis (Matasubara *et al.*, J. Immun., 137:3295-98 (1986)). Matasubara *et al.* have proposed that O₂⁻ and H₂O₂ function as “life signals”, preventing cells from undergoing apoptosis. (Matasubara *et al.*, J. Immun., 137:3295-98 (1986)). Other data suggest that ROI mediate both pro-apoptotic and pro-survival signals. (Garg & Aggarwal, Mol. Immunol., 39:509-17 (2002)).

NOX enzymes

A series of overexpression studies using the NOX1 enzyme of the NOX family of proteins indicates a specific role for NOX1-derived ROI in pathological conditions related to cell growth and proliferation. For example, overexpression of NOX1 in fibroblasts induces an H₂O₂-dependant malignant transformation, resulting in a highly tumorigenic phenotype (Arnold *et al.*, Proc. Natl. Acad. Sci. USA, 89:5550-55 (2001); Suh *et al.*, Nature (London), 401:79-82 (1999)). Consistent with these findings, overexpression of NOX1 in prostate epithelial cells has been found to increase tumorigenicity. This increased epithelial cell tumorigenicity is also associated with increased tumor vascularity and increased expression of vascular endothelial growth factor (VEGF), indicating a specific role for NOX1 in angiogenesis. (Arbiser *et al.*, Proc. Natl. Acad. Sci. USA, 99:715-20 (2002)).

One specific pathophysiological condition that may involve ROI is colorectal cancer (CRC), a form of cancer highly prevalent in the Western world. Sporadic colorectal cancer, which accounts for approximately 85% of diagnosed CRC, is linked to somatic mutations in the tumor suppressor gene adenomatous polyposis coli (APC). Genetic analyses of adenoma-carcinoma sequences for CRC indicate that mutations in APC are common, and may be the triggering event for the disease. The identification of a role for APC in CRC arises from the discovery of germline mutations in APC that result in the rare inherited form of colorectal cancer, familial adenomatous polyposis (FAP). Even though APC mutations may serve as a trigger for CRC, the fact that families carrying identical mutations in APC often exhibit varying degrees of colorectal cancer, both in severity and onset, suggests that other factors influence the function of APC. In other words, although a mutational loss of APC function may predispose an individual to colon cancer, other factors, such as ROI, may ultimately determine the onset and severity of CRC.

Duox enzymes

Dual oxidases, or Duox, have both a peroxidase-homology domain and a gp91*phox* domain. It is currently believed that Duox enzymes have dual enzymatic functions, catalyzing both the generation of superoxide and peroxidative type reactions. The latter class of reactions utilizes hydrogen peroxide as a substrate. Since hydrogen peroxide is generated spontaneously from the dismutation of superoxide, it is believed that the NAD(P)H oxidase domain generates the superoxide and/or hydrogen peroxide which can then be used as a substrate for the peroxidase domain. The peroxidase domain is likely to confer additional biological functions. Depending upon the co-substrate, peroxidases can participate in a variety of reactions including halogenation such as the generation of hypochlorous acid (HOCl) by myeloperoxidase and the iodination of tyrosine to form thyroxine by thyroid peroxidase. Peroxidases have also been documented to participate in the metabolism of polyunsaturated fatty acids, and in the chemical modification of tyrosine in collagen. Duox is also theorized to function in the formation or modification of extracellular matrix or basement membrane. Since the extracellular matrix plays an important role in tumor cell growth, invasion and metastasis, it is believed that the Duox type enzymes play a pathogenic role in such conditions.

Although a strong link exists between NOX and Duox enzymes and ROI function in a multitude of different physiological and pathophysiological conditions, *in vivo* models to study this link in a tissue-specific fashion are lacking. Without such models, the extent to which NOX or Duox-generated ROI participate in cellular proliferation and activation of growth-related signaling pathways in different tissue types is difficult to ascertain. Similarly, the identification of NOX or Duox enzyme regulatory molecules is difficult in the absence of such models.

Accordingly, what is needed are *in vivo* and *in vitro* models to examine the effect of NOX and Duox-derived ROI on cell proliferation and

activation of growth-related signaling pathways. Additionally, what is needed are *in vivo* and *in vitro* models to examine the effect of NOX and Duox-derived ROI on cell proliferation and activation of growth-related signaling pathways in a tissue specific manner. Also needed are *in vivo* and *in vitro* models to identify the regulators of NOX and Duox activity, including models to test the ability of different compounds to modulate the function of NOX and Duox and the effect of NOX and Duox-derived ROI on cellular proliferation and activation of growth-related signaling pathways.

10 SUMMARY

The present invention provides a transgenic non-human mammal, in particular a transgenic mouse, comprising a transgene encoding a member of the *gp91phox* homologue family of nicotinamide adenine dinucleotide phosphate-reduced form (NADPH) oxidase (NOX) and dual oxidase (Duox) proteins. The present invention also provides useful *in vivo* and *in vitro* models to examine the role of NOX and Duox proteins in the modulation of diseases and pathological conditions related to cell growth and proliferation.

The NOX and Duox family of proteins comprises NOX1 (SEQ ID NO:2), NOX2 (same as *gp91phox*) (SEQ ID NO:4), NOX3 (SEQ ID NO:6), NOX4 (SEQ ID NO:8), NOX5 (SEQ ID NO:10), Duox1 (SEQ ID NO:12) and Duox2 (SEQ ID NO:14) or modifications thereof. This family of proteins participates in the generation of reactive oxygen intermediates (ROI) and plays a role in several pathological conditions related to cell growth and proliferation, including, but not limited to, cancer, psoriasis, prostatic hypertrophy, benign prostatic hypertrophy, cardiovascular disease, proliferation of vessels, including but not limited to, blood vessels and lymphatic vessels, arteriovenous malformation, vascular problems associated with the eye, atherosclerosis, hypertension, and restenosis following angioplasty.

The present invention additionally provides DNA constructs comprising polynucleotides encoding a NOX or Duox polypeptide. In addition to NOX or Duox polypeptides, the invention also provides DNA constructs comprising polynucleotides encoding human NOX or Duox polypeptides and also NOX or Duox polypeptides from other species. DNA constructs containing the polypeptides can be incorporated into vectors for propagation or transfection into appropriate cells to generate NOX or Duox mutant non-human animals. The vectors may contain regulatory elements that provide for tissue specific or inducible expression of the nucleic acids and for expression of selectable markers for identification of the transfected cells. The present invention also comprises cells transfected with these DNA constructs, either in culture or integrated into the genome of the transgenic animal.

The present invention further provides methods for *in vivo* and *in vitro* identification of therapeutic agents, for example by screening chemical and drug libraries for compounds that alter the activity of the NOX or Duox enzymes. Such chemicals and drugs would likely be useful as treatments for the cellular proliferative disorders and diseases described above.

Accordingly it is an object of the invention to provide a transgenic non-human animal, which expresses NOX or Duox enzymes.

A further object of the invention is to provide a transgenic mouse which expresses NOX or Duox enzymes.

It is another object of the invention to provide DNA constructs encoding for NOX or Duox enzymes.

It is a further object of the invention to provide a non-human animal model system expressing NOX or Duox enzymes.

Yet another object of the invention is to provide a transgenic non-human animal comprising a transgene which encodes for NOX or Duox enzymes.

A further object of the invention is to provide a transgene comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 or SEQ ID NO:13.

Yet another object of the invention is to provide a transgene that
5 encodes for SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12 or SEQ ID NO:14.

It is an additional object of the invention to provide a non-human animal model system to study the *in vivo* and *in vitro* regulation and effects of NOX and Duox enzymes in specific tissue types.

10 It is a further object of the invention to provide a non-human animal model system to examine the role of NOX and Duox proteins in the generation of ROI in specific tissues.

Yet another object of the invention is to provide a non-human animal model system to examine the role of Nox proteins, particularly NOX1
15 in colon tissue.

A further object of the invention is to provide a non-human animal model system which has a propensity for the development of pathological conditions related to cell growth and proliferation.

Another object of the present invention is to provide a non-
20 human animal model system wherein the role of NOX and Duox-derived ROI on cell signaling in human and non-human animals may be examined in a tissue-specific manner.

It is yet another object of the invention to provide a transgenic animal useful for developing therapies for pathophysiological conditions
25 characterized by abnormal cellular proliferation.

It is another object of the present invention to provide a method for screening compounds for use in treating and preventing cellular proliferation disorders, particularly cancer.

Other objects, advantages and features of the invention will become apparent upon consideration of the following detailed description of the invention.

5 BRIEF DESCRIPTION OF THE FIGURES

This patent contains at least one color photograph. The U.S. Patent and Trademark Office will provide copies of this patent with the color photographs upon request and payment of the necessary fee.

Figure 1 is a diagram of the vector design for the development of conditional,
10 tissue-specific NOX1 overexpressing transgenic mice.

Figure 2: (A) is a photograph of dihydroethidium fluorescence in a cross-section of the colon of a wild type Black/6 mouse; (B) is a photograph of dihydroethidium fluorescence in a cross-section of the colon of a hNOX1 expressing Bl/6 mouse.

15 Figure 3: (A) is a photograph of one of two tumors found in the colon of an $Apc^{min}/h\text{-NOX1}$ mouse; (B-D) are photographs of histological analyses of the tumor shown in (A) by hematoxylin and eosin staining; (E) is a photograph of the adjacent section of normal colon.

Figure 4 is a graph of colon crypt pit to tip depth of NOX1 overexpressing
20 mice exposed to *Citrobacter rodentium* (*C. Rodentium*).

DETAILED DESCRIPTION OF INVENTION

The present invention provides a transgenic non-human animal, in particular a transgenic mouse, comprising a transgene encoding for a
25 member of the NOX and Duox family of proteins. The present invention further comprises methods and compositions for evaluating regulators of abnormal cell growth. The present invention additionally comprises methods and compositions for the development of compounds, such as drugs or other therapies, for the treatment of conditions associated with abnormal cell
30 growth, including, but not limited to, cancer, psoriasis, prostatic hypertrophy,

benign prostatic hypertrophy, inflammatory bowel disease, cardiovascular disease, proliferation of vessels, including but not limited to blood vessels and lymphatic vessels, arteriovenous malformation, vascular problems associated with the eye, atherosclerosis, hypertension, and restenosis following
5 angioplasty.

Reactive oxygen intermediates (ROI) generated in many non-phagocytic cells are now thought to originate from NOX or Duox enzymes. These enzymes are homologs of gp91*phox*, the catalytic subunit of the phagocyte NADPH oxidase. The NOX/Duox family consists, in humans, of 7
10 unique proteins NOX1 (SEQ ID NO:2), NOX2 (same as gp91*phox*) (SEQ ID NO:4), NOX3 (SEQ ID NO:6), NOX4 (SEQ ID NO:8), NOX5 (SEQ ID NO:10), Duox1 (SEQ ID NO:12) and Duox2 (SEQ ID NO:14). The sequences for the NOX and Duox family have been previously disclosed in WO/0028031, WO/0187957, and WO/02081703, each of which is
15 incorporated by reference in its entirety.

Each of the NOX or Duox enzymes has a specific expression profile in different tissues. For example, NOX1 is highly expressed in colonic epithelium, while NOX4 is highly expressed in kidney epithelium. While not wishing to be bound to any particular theory, it is believed that the proteins
20 related to gp91*phox* and involved in ROI generation in cells have been located in the NOX and Duox family of proteins. (Lambeth *et al.* (2001) *Gene* May 16; 269 (1-2):131-40; Edens *et al.* (2001) *J. Cell Biol.* Aug 20: 154(4):879-91; Lambeth *et al.* (2000) *Trends Biochem Sci.* Oct 25, (10); 459-61).

25 *Transgenes*

A transgene is a segment of DNA that has been incorporated into a host genome or is capable of autonomous replication in a host cell and is capable of causing the expression of one or more cellular products. Exemplary transgenes provide the host cell, or animals developed therefrom,
30 with a novel phenotype relative to the corresponding non-transformed cell

or animal. The NOX or Duox polynucleotides comprising the transgene of the present invention include NOX or Duox cDNA and can also include modified NOX or Duox cDNA. As used herein, a "modification" of a nucleic acid can include one or several nucleotide additions, deletions, or substitutions with respect to a reference sequence. A modification of a nucleic acid can include substitutions that do not change the encoded amino acid sequence due to the degeneracy of the genetic code, or which result in a conservative substitution. Such modifications to the nucleic acids can correspond to variations that are made deliberately, such as the addition of a poly A tail, or variations which occur as mutations during nucleic acid replication.

Exemplary modifications of the NOX or Duox nucleotide sequences include sequences that correspond to homologs of the nucleotide sequences for NOX1 (SEQ ID NO:1), NOX2 (SEQ ID NO:3), NOX3 (SEQ ID NO:5), NOX4 (SEQ ID NO:7), NOX5 (SEQ ID NO:9), Duox 1 (SEQ ID NO:11), Duox 2 (SEQ ID NO:13), such as homologs of other species, including mammalian species such as mice, primates, including monkey and baboon, rats, rabbits, bovine, porcine, ovine, canine, feline, or other animal species. The corresponding NOX or Duox sequences of non-human species including, but not limited to, *C. elegans* and *Drosophila* can be determined by methods known in the art, such as by PCR or by screening genomic, cDNA or expression libraries.

As employed herein, the term "substantially the same nucleotide sequence" refers to DNA having sufficient identity to the reference polynucleotide, such that it will hybridize to the reference nucleotide under moderately stringent, or higher stringency, hybridization conditions. DNA having "substantially the same nucleotide sequence" as the reference nucleotide sequence can have at least 60%, at least 70%, at least 80%, at least 90%, or at least 95% identity with respect to the reference nucleotide sequence.

The phrase "moderately stringent hybridization" refers to conditions that permit a target-nucleic acid to bind a complementary nucleic acid. The hybridized nucleic acids will generally have at least about 60% identity, at least about 70% identity, at least about 80% identity, at least about 90% identity, or at least about 95% identity. Moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5x Denhart's solution, 5x saline sodium phosphate EDTA buffer (SSPE), 0.2% SDS (Aldrich) at about 42°C, followed by washing in 0.2x SSPE, 0.2% SDS (Aldrich), at about 42° C.

High stringency hybridization refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at about 65° C. For example, if a hybrid is not stable in 0.018M NaCl at about 65° C, it will not be stable under high stringency conditions, as contemplated herein. High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5x Denhart's solution, 5x SSPE, 0.2% SDS at about 42° C, followed by washing in 0.1x SSPE, and 0.1% SDS at about 65° C.

Other suitable moderate stringency and high stringency hybridization buffers and conditions are well known to those of skill in the art and are described, for example, in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainview, N.Y. (1989); and Ausubel *et al.* (Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999)).

The amino acid sequences encoded by the transgenes of the present invention can be a NOX or Duox sequence, such as NOX1 (SEQ ID NO:2), NOX2 (same as gp91*phox*) (SEQ ID NO:4), NOX3 (SEQ ID NO:6), NOX4 (SEQ ID NO:8), NOX5 (SEQ ID NO:10), Duox1 (SEQ ID NO:12) and Duox2 (SEQ ID NO:14) or the NOX or Duox homologue from any species. The protein polypeptide sequence encoded by the transgene of the present invention can also be a fragment of the NOX or Duox amino acid sequence,

so long as the fragment retains some or all of the function of the full-length NOX or Duox sequence. The sequence may also be a modified NOX or Duox sequence. Individual substitutions, deletions or additions, which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 10%, more typically less than 5%, more typically less than 1%). A “modification” of the amino acid sequence encompasses conservative substitutions of the amino acid sequence. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Other minor modifications are included within the sequence so long as the polypeptide retains some or all of the structural and/or functional characteristics of a NOX or Duox polypeptide. Exemplary structural or functional characteristics include sequence identity or substantial similarity, antibody reactivity, the presence of conserved structural domains such as RNA binding domains or acidic domains, and the ability to generate ROI.

DNA Constructs and Vectors

The invention further provides a DNA construct comprising the NOX transgene or Duox transgene as described above. As used herein, the term "DNA construct" refers to a specific arrangement of genetic elements in a DNA molecule. In addition to human NOX or Duox, or mutant forms thereof, the invention also provides DNA constructs comprising NOX or Duox polypeptides from other species, as well as NOX or Duox mutant non-

human mammals expressing NOX or Duox from non-human species. The constructs further comprise a promoter or other regulatory element such as lox-P, and may additionally comprise a reporter or marker element such as green enhanced fluorescent protein (EGFP).

5 If desired, the DNA constructs can be engineered to be operably linked to appropriate expression elements such as promoters or enhancers to allow expression of a genetic element in the DNA construct in an appropriate cell or tissue, for example, a colon epithelial cell-specific promoter or a prostate epithelial cell-specific promoter. Cell and tissue specific promoters
10 are known to one of skill in the art. In one embodiment, the DNA construct may comprise a NOX or Duox polynucleotide sequence located upstream from two LoxP sites that comprise a “flox stop cassette.” Specific, non-limiting examples of loxP include, but are not limited to, the sequence listed as Genbank accession No. M10494.1; LOX P (Genbank Accession No.
15 U51223, herein incorporated by reference); LOX 511 (Bethke and Sauer, Nuc. Acid. Res. 25:282-34, 1997); ψ LOXh7q21 (Thyagarajan *et al.*, Gene, 244:47-54, 2000), ψ Coreh7q21 (Thyagarajan *et al.*, Gene, 244:47-54, 2000) as well as the Lox sites disclosed in Table 1 of Thyagarajan *et al.* (Gene, 244:47-54, 2000). Each loxP site corresponds to a 34 bp sequence, consisting of two 13-
20 bp palindromic sequences with an 8 bp central core. Within the “flox stop cassette” is located an EGFP gene sequence or other markers. Located upstream from the “flox stop cassette” is a tissue-specific promoter (such as a CX1 promoter). The use of the “flox stop cassette” and similar expression control mechanisms allows for the targeted delivery and expression of the
25 gene of interest.

The DNA constructs described herein may be incorporated into vectors for propagation, or transfection into appropriate cells to generate NOX or Duox mutant non-human mammals. The DNA constructs may also be incorporated into vectors for transfection into appropriate cells for the
30 development of cell cultures. One skilled in the art can select a vector based

on desired properties, for example, for production of a vector in a particular cell such as a mammalian cell or a bacterial cell.

The invention also provides vectors containing a NOX or Duox encoding polynucleotide sequence. Suitable expression vectors are well-known in the art and include vectors capable of expressing nucleic acids operably linked to a regulatory sequence or element, such as a promoter region or enhancer region, that is capable of regulating expression of such nucleic acids. Appropriate expression vectors include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those that integrate into the host cell genome and are known to one of ordinary skill in the art. For example, NOX1 with SV40 poly A can be subcloned into the HindII and Sac I sites of a vector containing a CX1 promoter.

Vectors can contain a regulatory element that provides tissue specific or inducible expression of an operably linked nucleic acid. One of ordinary skill in the art can readily determine an appropriate tissue-specific promoter or enhancer that allows expression of NOX or Duox polypeptides in a desired tissue. It should be noted that tissue-specific expression as described herein does not require a complete absence of expression in tissues other than the preferred tissue. Instead, "cell-specific" or "tissue-specific" expression refers to a majority of the expression of a particular gene of interest in the preferred cell type or tissue.

Any of a variety of inducible promoters or enhancers can also be included in the vector for expression of a NOX or Duox polypeptide or nucleic acid that can be regulated. Such inducible systems are known to one of ordinary skill in the art and include, for example, a tetracycline inducible system (Gossen & Bizard, Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992); Gossen *et al.*, Science, 268:1766-1769 (1995); Clontech, Palo Alto, Calif.); metallothionein promoter induced by heavy metals; insect steroid hormone systems responsive to ecdysone or related steroids such as muristerone (No *et al.*, Proc. Natl. Acad. Sci. USA, 93:3346-3351 (1996); Yao *et al.*, Nature,

366:476-479 (1993); Invitrogen, Carlsbad, Calif.); mouse mammary tumor virus (MMTV) induced by steroids such as glucocorticoid and estrogen (Lee *et al.*, Nature, 294:228-232 (1981); and, heat shock promoters inducible by temperature changes.

5 Regulatory elements, including promoters or enhancers, can be constitutive or regulated, depending upon the nature of the regulation, and can be regulated in a variety of tissues, a few specific types of tissues, or a single tissue type. The regulatory sequences or regulatory elements are operably linked to one of the polynucleotide sequences of the invention such that the
10 physical and functional relationship between the polynucleotide sequence and the regulatory sequence allows transcription of the polynucleotide sequence. Vectors useful for expression in eukaryotic cells can include, for example, regulatory elements including the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-
15 inducible promoter, Moloney murine leukemia virus (MMLV) promoter, CX1 promoter and the like.

 If desired, the vector can contain a selectable marker. As used herein, a "selectable marker" refers to a genetic element that provides a selectable phenotype to a cell in which the selectable marker has been
20 introduced, such as the fluorescence of the EGFP protein. A selectable marker is generally a gene whose gene product provides resistance to an agent that inhibits cell growth or kills a cell. A variety of selectable markers, generally known to one of ordinary skill in the art, can be used in the DNA constructs of the invention, including, for example, Neo, Hyg, hisD, Gpt and Ble genes, as
25 described, for example in Ausubel *et al.* (Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999)) and U.S. Patent No. 5,981,830. Drugs useful for selecting for the presence of a selectable marker include, for example, G418 for Neo, hygromycin for Hyg, histidinol for hisD, xanthine for Gpt, and bleomycin for Ble (see Ausubel *et al.*,
30 *supra*, (1999); U.S. Patent No. 5,981,830). DNA constructs of the

invention can incorporate a positive selectable marker, a negative selectable marker, or both (see, for example, U.S. Patent No. 5,981,830).

Non-Human Transgenic Animals

5 The present invention provides a nonhuman transgenic animal whose genome comprises a transgene encoding a NOX or Duox polypeptide. The transgene can be integrated into the genome of a transgenic animal by any method known to those skilled in the art. The transgene containing the desired gene sequence can be introduced into pluripotent cells, such as
10 embryonic stem (ES) cells, by any method that will permit the introduced molecule to undergo recombination at its regions of homology. Techniques that can be used include, but are not limited to, calcium phosphate/DNA co-precipitates, microinjection of DNA into the nucleus, electroporation, bacterial protoplast fusion with intact cells, transfection, and polycations,
15 (e.g., polybrene, polyornithine, etc.) The DNA can be single or double stranded DNA, linear or circular. (See for example, Hogan *et al.*, Manipulating the Mouse Embryo: A Laboratory Manual Cold Spring Harbor Laboratory (1986); Hogan *et al.*, Manipulating the Mouse Embryo: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory (1994), U.S. Patent Nos. 5,602,299; 5,175,384; 6,066,778; 4,873,191 and 6,037,521; retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, Proc. Natl. Acad. Sci. USA 82:6148-6152 (1985)); gene targeting in embryonic stem cells (Thompson *et al.*, Cell 56:313-321 (1989)); electroporation of embryos (Lo, Mol Cell. Biol. 3:1803-1814 (1983)); and sperm-mediated gene
20 transfer (Lavitrano *et al.*, Cell 57:717-723 (1989))).
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For example, the zygote is a good target for microinjection, and methods of microinjecting zygotes are well known to those of skill in the art (see U.S. Patent No. 4,873,191). In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter, which allows
30 reproducible injection of 1-2 picoliters (pl) of DNA solution. The use of

zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host genome before the first cleavage (Brinster, *et al.*, Proc. Natl. Acad. Sci. USA 82:4438-4442 (1985)). As a consequence, all somatic cells of the transgenic non-human animal will carry the incorporated transgene. This will in general, also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. The injected zygotes are transplanted to the oviducts/uteri of pseudopregnant females and finally transgenic animals are obtained.

Embryonal cells at various developmental stages can be used to introduce transgenes for the production of transgenic animals. Different methods are used depending on the stage of development of the embryonal cell. Such transfected embryonic stem (ES) cells can thereafter colonize an embryo following their introduction into the blastocoele of a blastocyst-stage embryo and contribute to the germ line of the resulting chimeric animal (reviewed in Jaenisch, Science 240:1468-1474 (1988)). Prior to the introduction of transfected ES cells into the blastocoele, the transfected ES cells can be subjected to various selection protocols to enrich the proportion of ES cells that have integrated the transgene if the transgene provides a means for such selection. Alternatively, PCR can be used to screen for ES cells that have integrated the transgene.

Retroviral infection can also be used to introduce transgenes into a non-human animal. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Janenich, Proc. Natl. Acad. Sci. USA 73:1260-1264 (1976)). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan *et al.*, supra, 1986). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner *et al.*, Proc. Natl. Acad. Sci. USA 82:6927-6931 (1985); Van der Putten *et al.*, Proc. Natl. Acad. Sci. USA

82:6148-6152 (1985)). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*, 1985; Stewart *et al.*, EMBO J. 6:383-388 (1987)). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner D. *et al.*, Nature 298:623-628 (1982)). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of cells which form the transgenic animal. Further, the founder can contain various retroviral insertions of the transgene at different positions in the genome, which generally will segregate in the offspring. In addition, transgenes may be introduced into the germline by intrauterine retroviral infection of the midgestation embryo (Jahner *et al.*, *supra*, 1982). Additional means of using retroviruses or retroviral vectors to create transgenic animals known to those of skill in the art involves the micro-injection of retroviral particles or mitomycin C-treated cells producing retrovirus into the perivitelline space of fertilized eggs or early embryos (WO 90/08832 (1990); Haskell and Bowen, Mol. Reprod. Dev. 40:386 (1995)).

Once the founder animals are produced, they can be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic mice to produce mice homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; breeding animals to different inbred genetic backgrounds so as to examine effects of modifying alleles on expression of the transgene and the effects of expression.

Crossbreeding of the transgenic mice can also allow for tissue specific expression of the transgene. Thus, while the NOX1 gene would exist in all somatic cells of the transgenic animal, expression of the NOX1 protein could be localized to, for example, the colon.

5 An example of a crossbreeding strategy for effecting tissue specific expression of NOX or Duox is through the use of the target recombining site sequence recognized by the bacterial *Cre* recombinase (*Cre*) which is available in mouse lines which have been previously developed to express *Cre* in a tissue specific manner. Homologous recombination is used
10 to insert loxP DNA sites into inactive regions of DNA flanking a marker EGFP gene and its associated stop sequence in the empty vector. NOX or Duox is then cloned into the empty vector upstream of the 3' loxP site to create a transgenic construct which is used to create a transgenic mouse. The resulting NOX or Duox mice are then crossed with Fabp4x at -132/*Cre*- mice
15 hemizygous for Fabp4x at -132/ *Cre* (*Cre* mice) (Saam, J. Biological Chemistry, 274:38071-38082 1999)(a gift from Jeffery I. Gordon (Washington University School of Medicine)). The *Cre* mice contain: (i) Fabp4x at -132 (nucleotides -596 to +21 of the rat Fabp4 gene, with four additional tandem repeats of its nucleotides -172 to -133 added at nucleotide -
20 132) linked to (ii) a 1.0 kb fragment, containing the *Cre* recombinase gene with a nuclear localization signal from SV40 large T antigen (from Gail Martin, University of California, San Francisco), and (iii) nucleotides +3 to +2150 of the human growth hormone gene (hGH). The P1 bacteriophage derived enzyme, *Cre* recombinase, then catalyzes recombination between the
25 two loxP sites, resulting in the excision of the reporter EGFP and its associated stop sequence. The loss of EGFP and its corresponding fluorescence in the transgenic offspring serves to verify *Cre*-mediated excision of the EGFP stop sequence; the excision of the stop sequence allows NOX or Duox to be expressed in the tissues of the transgenic mice. When the
30 two mouse lines are crossed, offspring are generated in which the gene of

interest is overexpressed when the promoter of the gene used to confer expression is normally activated.

To increase expression, or development of a particular disease or condition, a mouse overexpressing Nox1, such as the offspring of two crossed mouse lines such as the *Cre*/NOX1 mouse described above, can be further crossed with mice that have a proclivity to particular disease states. For example, the NOX overexpressing mice of the present invention were crossed with the multiple intestinal neoplasia (Min) mouse. The Min mouse is available commercially from Jackson Labs, Bar Harbor, Maine. Heterozygous Min mice show multiple gastrointestinal neoplasias, mainly small flat polyps in the small intestine. The mutation in the Min mouse is a nonsense mutation in codon 850 of the murine adenomatous polyposis coli (APC) tumor suppressor gene. The Min and *Cre*/NOX1 cross produced mice that developed colon tumors by 100 days. The *Cre*/NOX1/Min crossed mouse produced several large (0.5 cm) carcinomas protruding into the lumen of the colon (See Figures 3 and 3A). The Min/*Cre*/NOX1 cross is therefore a novel model for colon cancer. Other suitable mice lines specific for particular diseases or conditions are known to those of skill in the art. The present invention provides transgenic non-human mammals that carry the transgene in all their cells, as well as animals that carry the transgene in some, but not all their cells, that is, mosaic animals.

The transgenic animals are screened and evaluated to select those animals having the phenotype of interest. Initial screening can be performed using, for example, Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals can also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of the suitable tissues can be evaluated immunocytochemically using

antibodies specific for a NOX or Duox as appropriate, or with a tag such as EGFP. The transgenic non-human mammals can be further characterized to identify those animals having a phenotype useful in methods of the invention. In particular, transgenic non-human mammals overexpressing NOX or Duox
5 can be screened using the methods disclosed herein. For example, tissue sections can be viewed under a fluorescence microscope for the presence of fluorescence, indicating the presence of the reporter gene. (See Figures 2A and 2B).

Another method for affecting tissue specific expression of the
10 NOX or Duox proteins is through the use of tissue-specific promoters. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.*, (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and
15 Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.*, (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddell (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund *et al.*, (1985) *Science* 230:912-916), cardiac specific expression (alpha myosin
20 heavy chain promoter, Subramaniam, *et al.*, *J Biol Chem* 266: 24613-24620, 1991), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Patent Application Publication No. 264,166).

The invention further provides an isolated cell containing a
25 DNA construct of the invention. The DNA construct can be introduced into a cell by any of the well-known transfection methods (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, Plainview, N.Y. (1989); Ausubel *et al.*, *supra*, (1999)). Alternatively, the cell can be obtained by isolating a cell from a mutant non-human mammal created
30 as described herein. Thus, the invention provides a transfected cell or a cell

isolated from a NOX or Duox mutant non-human mammal of the invention, in particular, a NOX or Duox mutant mouse. The cells can be obtained from a homozygous NOX or Duox mutant mouse or a heterozygous NOX or Duox mutant non-human mammal such as a mouse.

5

Assays and Identification of Therapeutic Agents

The methods and compositions of the present invention are particularly useful in the evaluation of regulators of NOX or Duox enzymes and for the development of drugs and therapeutic agents for the treatment and
10 prevention of cancer, particularly in blocking the progression of polyps to a cancerous stage. The methods and composition of the present invention are particularly useful for the development of drugs and therapeutic agents for the treatment of colon cancer.

Compounds useful as potential therapeutic agents can be
15 generated by methods well known to those skilled in the art, for example, well known methods for producing pluralities of compounds, including chemical or biological molecules such as simple or complex organic molecules, metal-containing compounds, carbohydrates, peptides, proteins, peptidomimetics, glycoproteins, lipoproteins, nucleic acids, antibodies, and the like, are well
20 known in the art and are described, for example, in U.S. Patent No. 5,264,563; Francis *et al.*, Curr. Opin. Chem. Biol. 2:422-428 (1998); Tietze *et al.*, Curr. Biol., 2:363-371 (1998); Sofia, Mol. Divers. 3:75-94 (1998); Eichler *et al.*, Med. Res. Rev. 15:481-496 (1995); the National Cancer Institute Database, and the like. Libraries containing large numbers of natural and synthetic
25 compounds also can be obtained from commercial sources. Combinatorial libraries of molecules can be prepared using well known combinatorial chemistry methods (Gordon *et al.*, J. Med. Chem. 37: 1233-1251 (1994); Gordon *et al.*, J. Med. Chem. 37: 1385-1401 (1994); Gordon *et al.*, Acc. Chem. Res. 29:144-154 (1996); Wilson and Czarnik, eds., Combinatorial

Chemistry: Synthesis and Application, John Wiley & Sons, New York (1997)).

The transgenic animal, cells from the transgenic animal, or cells transfected *in vitro* with the transgenes of the present invention can be used in a variety of screening assays. For example, any of a variety of potential agents suspected of causing or contributing to cellular proliferation, hyperplasia and inflammation, as well as the appropriate antagonists and blocking therapeutic agents, can be evaluated by administration to the transgenic animal, cells of the transgenic animal, or transfected cells, and assessing the effect of these agents upon the function and phenotype of the cells. The compounds can increase, decrease or fail to alter the cellular proliferation, hyperplasia or inflammation.

The methods of the invention advantageously use cells isolated from a homozygous or heterozygous NOX or Duox mutant non-human mammal, for example, endothelial cells, epithelial cells, or muscle cells to study the regulatory mechanisms of ROI production and cellular proliferation, and to test potential therapeutic compounds. The methods of the invention are also used with cells expressing NOX or Duox such as a transfected cell line.

A cell overexpressing NOX or Duox can be used in an *in vitro* method to screen compounds as potential therapeutic agents for treating cellular proliferative disorders or other disorders involved in ROI production. In such a method, a compound is contacted with a cell overexpressing NOX or Duox, either a transfected cell or a cell derived from a NOX or Duox mutant non-human animal, and screened for alterations in a phenotype associated with expression of NOX or Duox. For example, the administration of the compound at a dose or level known to one of skill in the art, can increase cellular proliferation, decrease cellular proliferation or leave it unchanged. The compound can additionally specifically increase ROI production, decrease ROI production or have no effect. The changes in ROI production

and cellular proliferation in the cellular assay and the transgenic animal can be assessed by methods well known to those skilled in the art.

A NOX or Duox fusion polypeptide such as NOX1-EGFP is particularly useful for such screening methods since the expression of NOX or Duox can be monitored by fluorescence intensity. Other exemplary fusion polypeptides include other fluorescent proteins, or modifications thereof, glutathione S transferase (GST), maltose binding protein, poly His, and the like, or any type of epitope tag. Such fusion polypeptides are detected, for example, using antibodies specific to the fusion polypeptides. The fusion polypeptides can be an entire polypeptide or a functional portion thereof so long as the functional portion retains desired properties, for example, antibody binding activity or fluorescence activity.

Additionally, the level or activity of the NOX or Duox proteins expressed by the transgenes of the present invention, including the activity of the proteins in response to the addition of potential therapeutic agents, are measured by methods which include, but are not limited to, cytochrome c reduction, Nitroblue tetrazolium reduction, and luminescence.

The invention further provides a method of identifying a potential therapeutic agent for use in treating cellular proliferative disorders such as cancer, particularly colon cancer. The method includes the steps of contacting a cell containing a DNA construct comprising polynucleotides encoding a NOX or Duox polypeptide with the potential therapeutic agent and screening the cell for decreased NOX or Duox production or enzymatic activity, thereby identifying a potential therapeutic agent for use in treating a cellular proliferative disease. The cell can be isolated from a transgenic non-human animal having nucleated cells containing the NOX or Duox DNA construct. The cell can also contain a DNA construct comprising a nucleic acid encoding a green fluorescent protein fusion, or other fusion polypeptide, with a NOX or Duox polypeptide.

Additionally, cells expressing a NOX or Duox polypeptide can be used in a preliminary evaluation to identify compounds as potential therapeutic agents having activity that alters a phenotype associated with NOX or Duox expression. As with *in vivo* screens using NOX or Duox mutant non-human mammals, an appropriate control cell is used to compare the results of the evaluation. The effectiveness of compounds identified by an initial *in vitro* evaluation using cells expressing NOX or Duox is further tested *in vivo* using the NOX or Duox mutant non-human mammals of the present invention, if desired. Thus, the invention provides methods of screening a large number of compounds using a cell-based assay, for example, using high throughput screening, as well as methods of further testing compounds as therapeutic agents in an animal model of cellular proliferative disorders. Additionally, the cellular proliferative conditions being treated can be aggravated, for example, by the addition of an inflammatory agent such as *C. rodentium* to further analyze the effectiveness of the potential therapeutic agents.

Compounds identified as therapeutic agents by methods of the invention are administered to an individual, for example, to prevent, inhibit or reverse cellular proliferation. One skilled in the art will know or can readily determine the alleviation of a sign or symptom associated with cellular proliferative disorders such as cancer.

For use as a therapeutic agent, the compound is formulated with a pharmaceutically acceptable carrier to produce a pharmaceutical composition, which is administered to a human or other animal. Pharmaceutically acceptable carriers are known to one of ordinary skill in the art and include, but are not limited to, water, sodium phosphate buffer, phosphate buffered saline, normal saline, physiological buffer, Ringer's solution or other physiologically buffered saline, another solvent or vehicle such as a glycol or glycerol, an oil such as olive oil, or an injectable organic ester. A pharmaceutically acceptable carrier can also contain physiologically

acceptable compounds that act, for example, to stabilize or increase the absorption of the therapeutic agent. One skilled in the art knows that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition.

All publications and patents mentioned herein are incorporated by reference in their entireties for the purpose of describing and disclosing, the constructs and methodologies which might be used in connection with the present invention. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following examples. These examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

15

EXAMPLE 1

Construction of transgenic mice

The NOX1 transgenic plasmid, human full-length cDNA of NOX1 (SEQ ID NO:1) with SV40 poly A, was subcloned into Hind III and Sac I sites of a vector containing a CX1 promoter, and LoxP-flanked enhanced green fluorescent protein (EGFP) gene cassette (a gift of Dr. Kathy Griendling). In this construct, NOX1 cDNA is located at the 3' end of the LoxP-flanked EGFP cassette. Before pronuclear injection, the NOX1 transgenic plasmid was digested with Xho I to release the cassette containing the CX1 promoter, the LoxP-flanked enhanced green fluorescent protein (EGFP) gene, and NOX1 cDNA. Pronuclear injection was conducted at the Transgenic Mice Core Facility at Emory University. Two lines of C57BL/6J mice containing the NOX1 transgene were created and verified by Southern Blot and PCR (see following).

30

EXAMPLE 2

Verification of mouse pedigree

Using a P³²-labeled 792bp fragment of NOX1
cggtttaccgctcccagcagaaggtgtgattaccaaggtgtatgcacccatccaaagtttgggaattgcagat
5 gaacaagcgtggcttcagcatggaagtggggcagtatatctttgtaattgccctcaatctctcctggaatgg
catccttttactttgacctctgctccagaggaagattcttctccattcatatccgagcagcaggggactggacaga
aaatctcataagggcttcgaacaacaattaccattcccaggattgaagtggatggctcctttggcacagcc
agtgaggatgtttccagtatgaagtggctgtgctgggtggagcaggaattggggtcaccccccttgcttctatctt
gaaatccatctggtacaaattccagtgtgcagaccacaacctcaaaacaaaaagatctatttctactggatctgc
10 agggagacaggtgccttttctggtcaacaacctgttgacttcctggaacaggagatggaggaattaggcaa
agtgggttttctaaactaccgtctcttctcaccggatgggacagcaatattgttggtcatgcagcattaaacttga
caaggccactgacatcgtgacaggtctgaacagaaaacctcctttgggagaccaatgtgggacaatgagtttt
ctacaatagctacctcccacccaagtctgtagtgggagttttcttatgtggccctcggactttggcaaagagcct
gcgcaaatgctgtcaccgatattccagtctggatcc (SEQ ID NO:15) Southern Blot was
15 carried out using standard procedures to determine the copy numbers of trans-
NOX1 gene. (See, e.g., Sambrook, J.; Fritsch, E.F.; Maniatis, T.; "Molecular
Cloning: a laboratory manual"; Cold Spring Harbor Laboratory Press).
Briefly, 30 µg of genomic DNA from tail bits were digested overnight with
400 units of Bam H1. In addition, 2 µg of the transgenic vector containing
20 Nox1 DNA (SEQ ID NO:1) was digested with 40 units of BamHI and
amounts containing 1, 5, 10, 20, and 100 copies of Nox1 (SEQ ID NO:1) were
loaded along with 10 µg of carrier wildtype mouse DNA onto the same gel as
the Nox1 transgenic mice DNA. The resultant fragments were then separated
on a 0.7% agarose gel by means of overnight electrophoresis and then
25 transferred overnight by means of a capillary blot to nitrocellulose. To ensure
efficient transfer of the DNA fragments to the nitrocellulose, the gel was
stained with 10 mg/ml ethidium bromide following the transfer. 2 µg of the
transgenic vector containing Nox1 DNA was digested with 40 units of BamHI
and amounts containing 1, 5, 10, 20, and 100 copies of Nox1 were loaded
30 along with 10 µg of carrier wildtype mouse DNA. Hybridization was

performed using the P³²-labeled 792bp fragment of NOX1 as a probe. Following hybridization, the membrane was washed once with 500 ml 2X SSPE, 1% SDS for 25 minutes at room temperature and then three times with pre-heated 0.2X SSPE, 0.2% SDS for 20 minutes at 65°C to 68°C before exposure to X-ray film. One NOX1 transgenic mouse contained one copy while another contained 20 copies.

EXAMPLE 3

Cross breeding of NOX1 mice and Cre mice

10 Fabpl4x at -132/ Cre- CX1/(loxP)-hNOX1-Bi-transgenic mice were produced by crossing hemizygous C57BL/6J CX1/(loxP)EGFP(loxp)-hNOX1 mice with hemizygous FVB/N Fabpl4x at -132/Cre mice. Two pedigrees of Fabpl4x at -132/ Cre/CX1/(loxP)-hNOX1 were used.

EXAMPLE 4

Maintenance of Transgenic Mice

15 All mice used in these examples were housed in microisolator cages under a strict light cycle (lights on at 0700 h and off at 1900 h). Mice were given a standard irradiated chow diet (Rodent diet 5001, Labdiet® Richmond, Indiana) ad libitum. Animals were maintained in a specified pathogen-free state. All C57BL/6J and FVB/N mice were hemizygous for their respective transgenes.

EXAMPLE 5

Identification of Transgenic Mice

25 Genomic DNA was extracted from tail bits of 21day old mice and used to identify the crossbred mice positive or negative for hNOX1 and or Fabpl-Cre using PCR. hNOX1 DNA sequences were identified using primer 1, 5'-GTG AGG ATG TTT TCC AGT ATG AAG (SEQ ID NO:16) and
30 primer 2, 5'-TGT CAA AGT TTA ATG CTG CAT GAC CA (SEQ ID

NO:17). Cycle conditions were 95.0°C for 1 min 30 sec, 35 cycles of 95.0°C for 30 sec, 62.0°C for 20 sec, 72.0°C for 45 sec, and a final extension of 72.0°C for 3 min. These primers produce a 300bp amplicon. The amplicons were visualized on a 1.5% agarose gel.

5 Fabpl-*Cre* was detected using forward and reverse primers *CreF* (5'-AAC TGA AGA TGT TCG CGA TTA TCT (SEQ ID NO:18) and *CreR* (5'-ACC GTC AGT ACG TGA GAT ATC TT (SEQ ID NO:19) Cycle conditions were: 94.0°C for 6 min, 40 cycles of 94.0°C for 1 min, 54.0°C for 30 sec, 72.0°C for 30 sec, and a final extension of 72.0°C for 7 min. These
10 primers produce a 350 bp amplicon in Fabpl-cre transgenic mice. The amplicons were visualized on a 1.5% agarose gel.

EXAMPLE 6

EGFP Reporter Gene Assay

15 Sections (6µm) from colon, spleen, brain, liver, kidney, small intestine, and muscle from wildtype mice and mice identified in Example 5 as positive for the hNOX1 transgene were viewed on a Nikon eclipse TS-100 fluorescent microscope using a Nikon (FITC) filter (B-2E/C). Wildtype tissue sections and their respective hNOX1 transgene sections were compared for
20 the presence of fluorescence. (See Figure 2A and 2B)

EXAMPLE 7

Immunohistochemistry

Freshly harvested tissue was embedded in TBS (Triangle
25 Biomedical Sciences, Durham, N.C.) tissue freezing medium. A cryostat was then used to make semi-thin (5-10 µm) sections of the embeded tissue. The tissue sections were fixed in 2% paraformaldehyde in 1X PBS and placed at 4°C for 30 min. The sections were then washed 2 times in 1X PBS. (5 min/wash). Wet paper towels were layered in the base of moist chamber and
30 the slides were placed in the chamber and allowed to reach room temperature.

Before the slides dried, they were layered with 50 μ l 1X PBS. Blocking was performed using 1% normal goat serum or 2% BSA and 0.1% Triton in PBS for 1 hour. The primary antibody (a polyclonal antibody raised in rabbit against hNOX1, Lampire Biological Laboratories, Inc.) was centrifuged for 2 min at 13,500 rpm at 4°C and diluted in wash buffer (0.1% Triton in 1X PBS) at 1:100. The PBS was removed by aspirating at one end of the section and introducing antibody solution at the opposite end. The slides were then incubated for 1-2 hr, at room temperature. The slides were washed 3 times in 1X PBS (5 min/wash). The secondary antibody (goat anti-rabbit IgG Alexa Fluor 555 fluorescent antibody, Molecular Probes) was centrifuged for 2 min; 13,500 rpm at 4°C and diluted in wash buffer at 1:1000. 50 μ l of the antibody/wash buffer solution was used to cover the sections on each slide. The slides were then incubated in a moist chamber for 1 hr at room temperature. Next, the slides were washed 3 times in 1X PBS (5 min/wash). The slides were mounted with Gelvatol and allowed to dry before viewing on a confocal microscope. Crypt depth (from base of crypt to tip of crypt) was measured by micrometry on sections of the distal-rectal region of mouse colon stained with H&E. The measures of ten well-oriented crypts per animal were recorded prior to and after exposure to cryptobacter as detailed in Example 10, below. Measurements were taken before genotyping of the animal. (See Figure 4)

EXAMPLE 8

RNA extraction and RT-PCR

Freshly dissected tissues were taken and RNA was isolated using TRIzol Reagent Total RNA Isolation Reagent (GibcoBRL; Protocols outlined in <http://microarray.mbg.jhmi.edu/Trizol.pdf>). For RT-PCR, cDNA was synthesized using Advantage RT-for-PCR Kit (Clontech). A 20 μ l reaction mixture containing Moloney-Murine Leukemia Virus, recombinant (MMLV) reverse transcriptase (Clontech, Palo Alto, CA) and random

hexamer primer mix (Clontech, Palo Alto, CA) was used for the synthesis reaction. PCR was performed using 1 μ l of the above synthetic cDNA with primer 1 (SEQ ID NO:16) and primer 2 (SEQ ID NO:17). The thermocycler program was: 95.0°C for 1 min 30 sec, 35 cycles of 95.0°C for 30 sec, 62.0°C for 20 sec, 72.0°C for 45 sec, and a final extension of 72.0°C for 3 min. PCR products were visualized on a 1.5% agarose gel.

EXAMPLE 9

Detection of Expressed Reactive Oxygen Species

10 Fresh mouse colons were frozen in Tissue-Tek® O.C.T. (Optimal Cutting Temperature) Compound from Fisher Scientific International, Inc. and sectioned in a cryostat at a thickness of 6-10 μ m. Sections were kept at -80°C until use. The sections were then brought to room temperature, and in the dark were covered with 200-1000 μ L of 10 μ M
15 dihydroethidium (DHE) in HANKS buffer. Following a 15-30 min incubation at 37°C at 5 % CO₂, the slices were washed twice in HANKS buffer then mounted in Fluoromount G. DHE staining was immediately visualized using a fluorescence/confocal microscope. The Kolmogorov-Smirnov test was used to determine the significance of differences between data sets.

20

EXAMPLE 10

Challenge of mice with bacterial strain

Bacteria (*Citrobacter rodentium*; Genomospecies 9, Schaver, DB J. Clin. Microbiol. 33:2064-2068 1995) were grown overnight in five 5 ml
25 cultures. The *C. rodentium* was then pelleted from the five pooled overnight cultures and 100 ml of 20% sucrose was added to the pellet. On the morning of day 1, chow was removed and the water was replaced with the 100 ml *C. rodentium* 20% sucrose solution. Groups of five mice of two different genotypes, the Cre/NOX1/Min crosses and a control group, were allowed to
30 drink for 24 hrs. On day 2 the amount of bacteria/sucrose solution was

measured and the amount consumed by each mouse calculated. Dilutions of the bacteria/sucrose solution were plated out on agar and incubated overnight and counted to determine colony-forming units/mouse. A daily record of each mouse's weight was recorded. All mice were sacrificed on day 10 by CO₂.

- 5 Four cm of the colon, measured from the rectum, was cleaned with 1X PBS, allowed to drain, weighed, frozen in O.C.T., and stored at -80°C until use.

EXAMPLE 11

Measurement of colon crypt depth

- 10 Colon crypt depth was measured from pit to tip (See Figure 4)
In the figure, each point represents a separate measurement, on 5 animals in each group. The four groups of mice were wild type, mice with the transgene for NOX1, as controls and mice with and without NOX1 exposed to *Citrobacter*. This depth is a measure of hyperplasia (or overgrowth of the
15 colonic epithelial cells). Hyperplasia is a response that happens normally upon exposure of mice to pathogenic bacteria, in this case *Citrobacter* in the drinking water (compare first set of points to third). This demonstrates that over-expression of NOX1 in colonic epithelium alone does not have much effect on hyperplasia (first 2 groups). However, upon exposure to pathogenic
20 bacteria, there is a marked increase in the hyperplastic response (last 2 groups). Therefore, reactive oxygen by itself is not sufficient for growth. However, in combination with another stimulus, such as *Citrobacter*, it has an amplifying effect, resulting in marked growth.

- All patents, publications and abstracts cited above are
25 incorporated herein by reference in their entirety. It should be understood that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the present invention as defined in the following claims.